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Phagolysosomal pH in Alveolar Macrophages

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We studied phagolysosomal pH in alveolar macrophages (AM) using fluorescein-labeled yeast (FYP) and silica particles (FSP) as probes. Fluorescence intensities from the ingested test particles were measured on populations of AM using fluorescence spectrometry and on individual phagolysosomes using fluorescence microscopy. Measurements were performed on rabbit AM, which had been incubated with FYP or FSP (*in vitro* procedure). We also instilled FYP or FSP via the trachea into rabbit lungs and after 1 day, 1 week, 1 month, and 3 months lavaged the lungs and measured the pH in AM (*in vivo* procedure). Phagolysosomal pH was independent of the number and size of the fluorescent particles. Measurements of populations of AM with fluorescence spectrometry and of individual phagolysosomes with fluorescence microscopy gave similar average pH. For the FYP, pH decreased during the first day after lavage both in the *in vitro* and the *in vivo* procedures. For the FSP, pH was unchanged during the same period. After 1 day pH was similar for both particles. Electron microscopy showed a larger number of lysosomes in contact with phagosomes and a higher percentage of vacuolated phagosomes for FYP than for FSP. In the *in vivo* procedure, pH was unchanged at least up to 1 month, and this pH was lower than that in the *in vitro* procedure. The difference was probably due to conditions at the time of phagocytosis. Particles retained in the lung parenchyma were within AM, and their location within the AM appeared unchanged from 1 week up to 3 months.

Introduction

Mechanical clearance of particles from the alveolar part of the human lung is extremely slow; the majority of the particles clear with half-times up to several years (*1–4*). However, alveolar macrophages can efficiently dissolve many inorganic particles with low solubility in water (*5–9*).

Studies of neutrophils and peritoneal macrophages from several species have demonstrated a varying but acid pH in phagolysosomes or lysosomes (*10–13*). This acid milieu is at least one factor of importance for affecting the ability of the macrophages to dissolve metal particles. This is strongly supported by the fact that alveolar macrophages dissolve lead arsenate particles at a faster rate than the culture medium but dissolve arsenic trisulfide particles at a slower rate than the medium, whereas solubility of lead arsenate in water increases and arsenic trisulfide decreases with decreasing pH (*9*). Furthermore, some of the particles used in the experiments with macrophages cited above showed a marked increase in dissolution rate in water with decreasing pH (*5,9*).

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Ohkuma and Poole (*12*) described a method for measuring pH in lysosomes using fluorescein-labeled dextran. In cell populations, using a fluorescence spectrometer, they estimated lysosomal pH from the relationship between the ratio of the fluorescence at wavelength 519 nm and excitation wavelengths 495 and 450 nm and pH. We used this principle for measurements of phagolysosomal pH in alveolar macrophages with fluorescein-labeled yeast particles (FYP) or fluorescein-labeled amorphous silica particles (FSP) as probes (*14–17*). The present paper reviews our studies with these particles concerning methodological aspects and includes examination of the influence of particle number, size, and material on pH, comparisons of pH obtained from the test particles *in vitro* and *in vivo*, as well as after various times after the administration of particles. Furthermore, pH obtained by fluorescence spectrometry on populations of macrophages was compared with pH obtained by measurements on individual macrophages by fluorescence microscopy.

Materials and Methods

Particles

Heat-killed yeast cells, length 5.0 ± 0.7 μm, width 3.5 ± 0.6 μ (mean ± SD) and amorphous silica particles, 3.0 ± 0.5 or 5.0 ± 0.6 μm in diameter, were labeled with fluorescein. For details, see Nilsen et al. (*14*) and Nyberg et al. (*15*). Figure 1 show standard curves for FYP and FSP obtained from fluorescense measurements of the particles suspended in series of Hank's balanced salt solution (BSS) with various pHs.

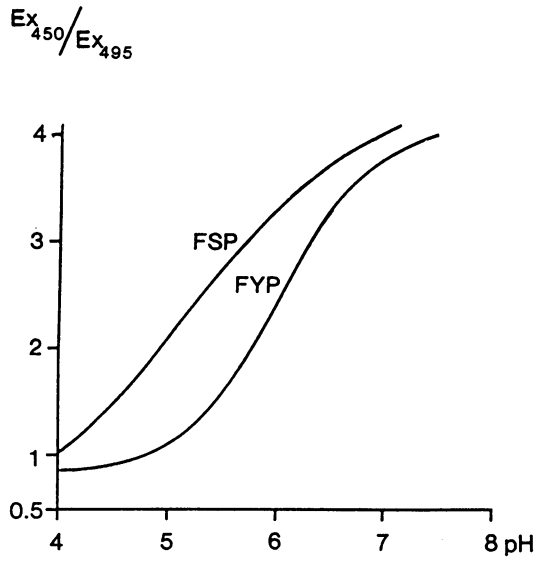

FIGURE 1. Relationships between fluorescein-labeled yeast particles (FYP) and fluorescein-labeled silica particles (FSP) and the ratio between the fluorescence intensities at 519 nm with excitation wavelength 495 nm (Ex_{495}) and 450 nm (Ex_{450}).

In Vitro Procedures

We lavaged alveolar macrophages from the lungs with Hank's BSS. The macrophage suspension was centrifuged and resuspended in medium 199 with 20% rabbit serum. After incubating the macrophages for 3 hr or longer at 37°C, we measured phagolysosomal pH. For details, see Nilsen et al. (*14*) and Nyberg et al. (*15,16*).

In Vivo Procedures

We intratracheally instilled rabbits with 2–3 × 10^8 test particles in 1 mL Ringer acetate or Hank's BSS. After 1 day, 1 week, 1 month, or 3 months, we killed the rabbits and collected the macrophages by lavage. The suspension was centrifuged and the macrophages resuspended in medium 199 with 20% rabbit serum, and the pH measurements were performed as for *in vitro* procedures. For details, see Nilsen et al. (*14*) and Nyberg et al. (*15,17*).

Fluorescence Measurements

In the measurements with fluorescence spectrometry, we mounted coverslips with macrophages in a holding device and placed them in a standard fluorescence cell with an angle of 30° to the exciting light beam. The measurements were performed with Hank's BSS in the fluorescence cells. We measured the fluorescence intensities at 519 nm at excitation wavelengths 495 and 450 nm. After the pH measurements, trypan blue was added. Because the intensities from the noningested particles were quenched by the trypan blue, the percentage of noningested particles could be determined. Usually, only samples where the percentage of noningested particles was <5% were accepted. For details, see Nilsen et al. (*14*) and Nyberg et al. (*15,16*).

For the measurements of fluorescence intensities from individual phagolysosomes, we used a technique for microscopic fluorometry described by Rundqvist and Enerback (*18*). The instrument was modified for intracellular pH measurements by inserting excitation filters, one with a transmission maximum at 452 nm and another with a transmission maximum at 489 nm. Fluorescence light above 515 nm was selected by a barrier filter. For details, see Nyberg et al. (*16*).

Location of Particles

Three sections from each of the right and left inferior lung lobes were prepared with a distance of 8 mm between the sections. From each section, we used a fluorescence microscope to find 100 particles and to determine whether these particles were located inside or outside macrophages. For details, see Nyberg et al. (*17*). Macrophages from rabbits that had been injected intratracheally with FSP 24 hr, 1 week, and 3 months earlier were examined with electron microscopy, and the intracellular location of FSP was studied. For details, see Nyberg et al. (*17*).

Results and Discussion

Influence of Particle Number and Size on pH

Table 1 presents results from studies in which different numbers of FYP and FSP were incubated with macrophages. The number of added particles was approximately proportional to the number of particles/macrophage, for example, 1 × 10^6 added FSP gave 0.6 particles/macrophage and 15 × 10^6 added FSP gave on the average 10 FSP/macrophage (*15*). Three- and 5-μm FSP gave almost identical pH (Table 1) (*15*). It is apparent from Table 1 that pH is independent of number and size of the particles.

Comparison of pH Obtained with Spectrometer and Microscope

The procedure to estimate phagolysosomal pH by spectrometric measurements of fluorescence intensities in populations of macrophages has two disadvantages. One is that both intracelluar and extracellular particles are measured. We have overcome this difficulty by counting extracellular and intracellular particles in the samples after the pH measurements and by accepting only samples with a low percentage of extracellular particles (<5%). The other difficulty is that the quotient of the fluorescent intensities is not linearly related to pH, but is relatively higher for high compared to low pH values in the pH interval of 4–6 (Fig. 1). This means that pH obtained using measurements

Table 1. Intraphagolysosomal pH obtained when different numbers of FYP and FSP were incubated with samples of 2–3 × 10^6 macrophages (*14,15*).

	Number of particles				
	1 × 10^6	2.5 × 10^6	5 × 10^6	10 × 10^6	15 × 10^6
FYP (mean ± SD of four rabbits)	5.6 ± 0.1	5.6 ± 0.2	5.7 ± 0.1	5.5 ± 0.1	5.6 ± 0.1
FSP (mean ± SD of five rabbits)	4.5 ± 0.1	4.7 ± 0.1	4.8 ± 1	4.8 ± 1	4.8 ± 1

Abbreviations: FYP, fluorescein-labeled yeast particles; FSP, fluorescein-labeled silica particles.

in a spectrometer may be overestimated. Table 2 compares phagolysosomal pH obtained from measurements of macrophage populations by fluorescence spectrometry with measurements on single particles in phagolysosomes by fluorescence spectrometry in samples from the same rabbits. Table 2 shows a good agreement between the pH values obtained with the two methods, apparently due to the rather small variation in pH among phagolysosomes in macrophages from the same cell sample with a coefficient of variation below 10%, often only around 5% (*16*).

Comparison of FSP and FYP

pH Estimation. In the measurements using FYP, there was a decrease in pH during the first day after lavage, both in the *in vitro* and the *in vivo* procedure, whereas in measurements with FSP, the pH was unchanged during the same period (*14,15*). Table 3 presents phagolysosomal pH using FYP and FSP in macrophages cultured for 3 and 24 hr. In macrophages cultured for 3 hr, pH is clearly higher for FYP than for FSP, but in macrophages cultured for 24 hr the values are about the same for FYP and FSP.

Table 2. Phagolysosomal pH in samples from three rabbits estimated from measurements on individual phagolysosomes in a fluorescence microscope and on populations of macrophages in a fluorescence spectrometer (*16*).

Hours after incubation	Rabbit	Microscopy	Spectrometry
3	1	5.3	5.2
	2	5.3	5.2
	3	5.4	5.5
6	1	5.2	5.2
	2	5.5	5.5
	3	5.3	5.4
24	1	5.1	5.2
	2	5.3	5.2
	3	5.2	5.3

Table 3. Phagolysosomal pH obtained using FYP and FSP in macrophages cultured for 3 and 24 hr (*16*).

	3 hr		24 hr	
Rabbit	FYP	FSP	FYP	FSP
1	5.4	4.9	5.1	5.1
2	6.1	4.9	5.0	4.9
3	5.5	5.1	5.1	4.9
Mean ± SD	5.8 ± 0.3	5.0 ± 0.1	5.0 ± 0.1	5.0 ± 0.1

Abbreviations: FYP, fluorescein-labeled yeast particles; FSP, fluorescein-labeled silica particles.

Table 4. Number of lysosomes in contact with the phagolysosomes and percentage of phagolysosomes in macrophages cultivated for 3 and 24 hr (*15*).[a]

	3 hr		24 hr	
	FYP	FSP	FYP	FSP
No. of lysosomes in contact with the phagolysosomes	6.1 ± 1.3	1.0 ± 0.1	3.2 ± 1.1	1.1 ± 0.2
% Vacuolated phagosomes	73 ± 18	7 ± 7	88 ± 6	12 ± 10

Abbreviations: FYP, fluorescein-labeled yeast particles; FSP, fluorescein-labeled silica particles.

[a]Data are given as means ± SD of six rabbits. About 50 particles/preparation were scored.

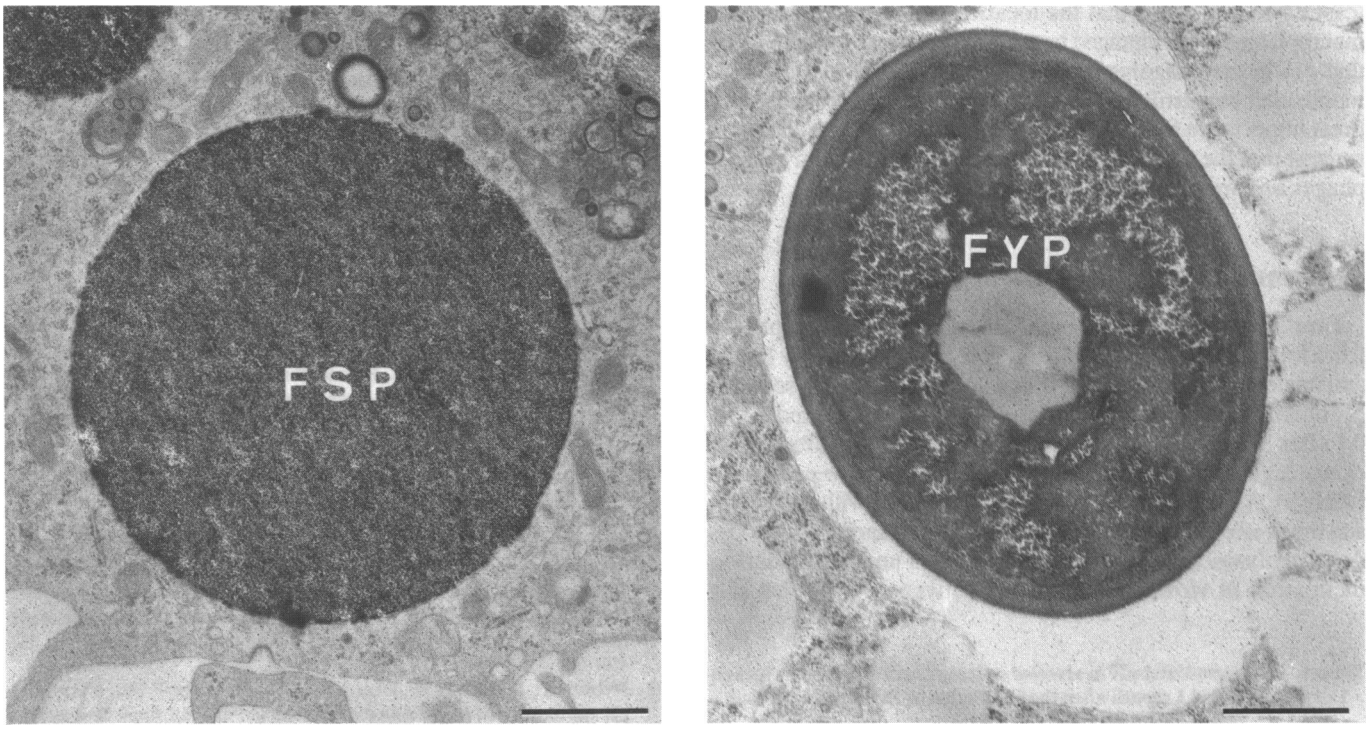

FIGURE 2. (*A*) Phagolysosome containing a silica particle (FSP), which is in tight contact with the phagolysosomal membrane. (*B*) Phagolysosome containing a yeast particle (FYP). There is about a 200 nm clear zone between the particle and membrane.

Morphology. Electron microscopic examinations of the location of FYP and FSP in macrophages cultured for 3 and 24 hr showed clear differences between the particles. The number of lysosomes in contact with phagolysosomes containing particles was higher for phagolysosomes with FYP than with FSP (Table 4). Furthermore, in phagolysosomes containing FYP, clear zones (vacuolization) were usually seen between the FYP and the phagolysosome membrane (Table 4, Fig. 2). Such a clear zone was usually not seen between the FSP and the phagolysosomal membrane.

Dependence with Time *in Vivo*

FSP was instilled into the trachea of the rabbits, and groups of four rabbits were killed and their lungs lavaged after 24 hr, 1 week, 1 and 3 months (*17*). Due to the marked decline of the fluorescence intensities from the FSP between 1 and 3 months, it was not possible to measure phagolysosomal pH 3 months after instillation. Table 5 shows that pH is highly similar 1 day, 7 days and 1 month after instillation of the FSP.

On the average, 94% of the FSP were located in macrophages after 1 day. After 1 week about 99% and after 1 and 3 months almost all particles were inside macrophages. In the electron microscopic examination of the location of the FSP, there was an increase in the number of lysosomes in contact with the phagolysosomes between 1 day and 1 week, but no change between 1 week and 3 months. There was also an increase in the percentage of vacuolated phagolysosomes with FSP between 1 day and 1 week, but there was no change between 1 week and 3 months.

Our results thus indicate that instilled particles that are retained in the lung parenchyma are located in alveolar macrophages at least up to 3 months, that the location of particles within the macrophages is not changed between 1 week and 3 months, and that pH in the phagolysosomes is unchanged at least up to 1 month after the instilltion. As phagolysosomal pH probably is of great importance for the dissolution of metal particles, our data suggest that the dissolution of particles by the alveolar macrophages *in vivo* is rather constant over time.

Comparison of pH *in Vitro* and *in Vivo*

Parallel to the 3-month *in vivo* study, alveolar macrophages from four rabbits not injected with FSP were obtained by lavage and incubated with FSP for 3, 6. and 24 hr (*17*). Phagolysosomal pH obtained was 5.4 ± 0.1 (mean ± SD), 5.4 ± 0.1., and 5.3 ± 0.1 after 3, 6. and 24 hr, respectively. These values are significantly higher than the values obtained in the *in vivo* procedure (Table 5). A similar difference in the pH obtained with the *in vitro* and the *in vivo* procedures was also found in an earlier study (*15*). As phagolysosomal pH obtained by FSP is unchanged during incubation *in vitro*, the difference between the *in vivo* and *in vitro* procedures must be due to the conditions of the cell and its environment at the time of the phagocytosis.

Table 5. Phagolysosomal pH in alveolar macrophages obtained by lavage 1 day, 7 days, and 1 month after the instillation of fluorescein-labeled silica particles (*17*).[a]

1 day	7 day	1 month
4.9 ± 0.2	4.8 ± 0.2	4.9 ± 0.1

[a]Data are given as means ± SD of four rabbits.

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